M E M O R A N D U M

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RE: Comments on 42 CFR Part 1003 (Possession, Use, and Transfer of Select Agents and Toxins; Interim Final Rule
Exclusion of attenuated strains of *Yersinia pestis*

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We are requesting that 42 CFR Part 1003 (Possession, Use and Transfer of Select Agents and Toxins; Interim Final Rule) be modified to exclude *Yersinia pestis* strains with two types of mutations from **some** of the regulations set forth in that document. This partial exemption is warranted since these are avirulent or highly attenuated strains that do not pose a severe threat to the public health and safety.

There are two deletion mutations in essential sets of virulence genes of *Y. pestis* that eliminate these strains as potential sources for bioterrorism/biowarfare and public health threats. The specific deletions that we are requesting for exclusion are: 1) a ~70 kb virulence plasmid encoding the low-calcium response (Lcr) that includes a secretion system and a set of virulence proteins that are essential for virulence; and 2) the 102 kb chromosomal pigmentation (*pgm*) locus that encodes an iron transport system that is essential for bubonic plague. The entire genomes of two biotypes of *Y. pestis* (biotype *orientalis* strain CO92 and biotype *mediaevalis* KIM) have been sequenced, analyzed, and annotated. In addition, both the *pgm* locus and Lcr plasmids from several other strains of *Y. pestis* have been sequenced. Thus, we know precisely the genes missing in these deletion mutants. In vitro and in vivo studies have defined the physiological and virulence roles of both the *pgm* locus and the Lcr plasmid.

1. Lcr

Y. pestis strains lacking the ~70-kb virulence plasmid are termed Lcr$^-$. Strains carrying this plasmid exhibit a dependence on calcium for growth at 37^{o}C; thus these bacteria are occasionally

referred to as Cal^+ in some of the literature. The plasmid encodes a set of virulence proteins called Yops and a protein called V antigen (virulence antigen; these strains are termed VW^+ or Vwa^+ in the older literature) as well as a special secretion system for these virulence proteins. Cited below are the reasons and evidence for excluding all Lcr^- strains of *Y. pestis* from the regulations described in 42 CFR Part 1003:

A. In the plague research community, loss of the Lcr property/plasmid has been accepted as causing "universal avirulence" (3, 4).

B. There is extensive experimental evidence that the Tjiwidej S strains (also called TS) and other Lcr^- strains of *Y. pestis* are avirulent in experimental animals (6, 12, 18). Attempts to recover virulent organisms from the Tjiwidej S strain failed, even after X-ray or UV-irradiation followed by animal passage (5). Most of these studies were done before the Lcr plasmid was discovered and it was realized that virulence could not be recovered since the essential virulence genes were missing in these mutants. However, the attempt to recover virulence of an Lcr^- strain is still important since it demonstrates that other mutations cannot compensate for loss of the Lcr plasmid.

C. Strain Tjiwidej S, which research literature suggests has all *Y. pestis* virulence determinants except Lcr, was used extensively as a live vaccine in humans in Java (15, 16). More than 2 million people received subcutaneous injections of cultures containing as many as 100 million viable *Y. pestis* Tjiwidej S bacteria plus 200 million dead bacteria with reactions comparable to typhoid vaccine of those days (14).

D. Lcr^- strains of *Y. pestis* that retain all the other virulence properties were studied for at least two decades (ca. 1960 to 1980) under BSL"0" containment conditions. In those days, these bacteria were mouth pipetted, sonicated, centrifuged (in liter quantities), and subjected to other procedures with a high aerosol potential (such as resuspension of pelleted 10-L fermentor-growths with a large syringe and 13 gauge cannula) without a single adverse consequence. Thus there is no or extremely minimal hazard posed from these strains from an aerosol route.

E. It is technically possible to restore virulence to an Lcr^- strain by re-introducing the virulence plasmid. However, in practice this is difficult, because the plasmid is not naturally transmissible, it is large, and does not have an easy selectable marker. Hence genetic engineering of the plasmid as well as special knowledge and equipment would be necessary to restore virulence to Lcr^- strains.

All of the above evidence indicates that Lcr^- strains of *Y. pestis* are completely and stably avirulent and thus "do not pose a severe threat to the public health and safety". Consequently, we request that Lcr^- strains of *Y. pestis* be exempted from the select agent safety and security regulations described in section 73.11, parts c3 and c4, and in section 73.15, parts b, c, and d.

2. Pgm

This property was named for the ability of wildtype *Y. pestis* strains to adsorb onto their surface low-molecular-weight planar molecules such as hemin and Congo red and hence appear "pigmented". Spontaneous mutants lost the hemin and Congo red adsorption characteristic and were avirulent in experimental animals unless the animals were injected with iron (11, 12). It is

now known that most of these spontaneous Pgm$^-$ mutants are due to deletion of a 102-kb region of the chromosome (9) termed the *pgm* locus. As for the Lcr plasmid, once deleted, this DNA cannot be regained. This locus includes an ~7 kb operon (*hmsHFRS*) that is required for hemin and Congo red adsorption; this Hms phenotype is required for transmission of the disease from fleas to mammals but is not important during the course of disease in mammals (13; reviewed in 17). The loss of virulence observed in experimental animals results from loss of an iron-acquisition system termed yersiniabactin (Ybt) (1, 8). Cited below are the reasons and evidence for excluding all *Δpgm* (*pgm* deletion) strains of *Y. pestis* from the regulations described in 42 CFR Part 1003:

A. Pgm$^-$ mutants possessing all the remaining virulence determinants of *Y. pestis* were isolated and shown to be avirulent (6, 12). Subsequent studies demonstrated that *Δpgm* mutants are completely avirulent in mice infected from peripheral routes (subcutaneous or peritoneal injection); however, such mutants are virulent when introduced intravenously in mice (2, 18). The fact that virulence is conditional has provided a biological containment that protects laboratory workers from contracting plague while allowing studies of pathogenesis in models of systemic (septicemic) plague.

B. An attempt to recover virulent *Y. pestis* by mouse passage of an avirulent Pgm$^-$ strain was unsuccessful (12). This demonstrates that other mutations cannot compensate for loss of the *pgm* region.

C. *Y. pestis* Strain E.V. (or various substrains such as EV 76) is Pgm$^-$ and was used for large-scale subcutaneous vaccination of humans in Madagascar without "the slightest mishap" (10, 14, 15).

D. As with Lcr$^-$ strains, Pgm$^-$ Lcr$^+$ strains of *Y. pestis* were studied for ca. 2 decades under BSL"0" containment conditions. Again, in the mid-60's, these bacteria were mouth pipetted, sonicated, centrifuged (in quantities up to 20 liters), and subjected to other procedures with a high aerosol potential (such as syringe-dispersal of huge bacterial pellets) without a single adverse consequence. For more than four decades, work has been done in multiple laboratories with Pgm$^-$ Lcr$^+$ strains of *Y. pestis* on the open bench, using standard microbiological practices without extra respiratory precautions, with no incidents of *Y. pestis*-induced illness. Thus there is negligible hazard posed by these strains to humans exposed from an aerosol route.

E. A recent study determined that the LD_{50} of a *Δpgm* strain by aerosol infection of mice was ~10^6 bacteria. The reason for the uncertainty in the LD_{50} calculation was not explained but the value of 10^6 represents a 42-fold loss of virulence compared to the parental Pgm$^+$ strain. The same study examined aerosol infection of African green monkeys with the same *Δpgm* strain and found that only 8 of 16 exposed monkeys died. An LD_{50} value could not be calculated since there was no correlation between the bacterial infecting dose and death. In addition, 5 of the 8 monkeys had significant delays in time to death (10-25 days) and 2 of the 6 monkeys examined had no culturable *Y. pestis* cells in their lungs or spleen. Finally 5 of the 8 surviving monkeys were exposed to inhaled doses of 10^6 or 10^7 bacteria (19). In a previous study by this same group, aerosol infection of 15 African green monkeys with the Pgm$^+$ parent strain caused the death of all monkeys even at the lowest dose of 140 bacteria. The average time-to-death was 5.6 days and *Y. pestis* cells were isolated from the lungs of all

monkeys and from the spleens of 14 of the 15 monkeys (7). Clearly the *Δpgm* mutation had a significant effect on both the virulence by the aerosol route and on the disease course of pneumonic plague. Finally, the fact that no *Y. pestis* cells could be cultured from some monkeys and these monkeys had an extended time-to-death (25 days) raises the possibility that some monkeys in this study may have died of something other than plague.

F. It would be extremely difficult to reconstitute virulence in a *Δpgm* strain of *Y. pestis*, and this has not been done by even the experts in the field. It is clear that one cause of the loss of virulence by subcutaneous and aerosol routes is the deletion of, and other mutations of, the siderophore-dependent Yersiniabactin (Ybt) iron transport system (1, 8). Essential *ybt* genes within the *pgm* locus encompass ~29 kb of DNA. The research of Drs. Perry and Fetherston has focused on this iron transport system and they have been unable to fully complement the deletion of these *ybt* genes by transforming a *Δpgm* strain with the *ybt* locus carried on a recombinant plasmid (unpublished observations). In addition, it is possible that one or more essential virulence genes are encoded within the remaining ~70 kb of DNA of the *pgm* locus.

Both laboratory experience for over 4 decades and experimental evidence indicate that *Δpgm* strains of *Y. pestis* are avirulent or extremely attenuated by any route other than intravenous injection. Thus they pose no significant threat to the public health. If used by bioterrorists, *Δpgm* strains of *Y. pestis* would cause no more harm than many other category B or C organisms that are not covered by the 42 CFR Part 1003 (Interim Final Rule). Consequently, we request that *Δpgm* strains of *Y. pestis* be exempted from the select agent safety and security regulations described in section 73.11, parts c3 and c4, and in section 73.15, parts b, c, and d.

3. Summary

Drs. Jacqueline D. Fetherston, Robert D. Perry and Susan C. Straley perform the majority of their research with Pgm^+ Lcr^- or Pgm^- Lcr^+ strains in their BSL2 laboratories (Medical Sciences Building MS341 for Perry and Fetherston; MN365/371 for Straley). No fully virulent or Pgm^+ Lcr^+ strains are kept in either laboratory. We believe there is strong evidence that neither Lcr^- nor Pgm^- deletion mutants pose a significant, let alone a severe, threat to public health. We are not requesting that these strains be exempted from all regulations described in 42 CFR Part 1003 (Interim Final Rule). Since it is technically possible to restore virulence to some of these mutants, it is prudent to adhere to the regulations in sections other than portions of section 73.11 and 73.15. In section 73.11 we are requesting exemption from section c3 and c4 which state that unlocked containers not in direct view of approved staff be locked at all times and that packages be inspected upon entry and exit from the area. Access to these laboratories which contain only avirulent/attenuated strains are already restricted to approved personnel. In section 73.15 we are requesting exemption from the stringent inventory regulations in sections 73.15b-d. This would retain registration for transfer of these mutants as well as increased security for access to the laboratories, approval of personnel for access to these strains, and personnel training without daily record-keeping on usage of literally hundreds of avirulent/highly attenuated mutants.

In addition to researchers at the University of Kentucky, a significant number of other researchers in the United States use one or both of these avirulent/attenuated strains in their research - Robert R. Brubaker (Michigan State Univ.), Stanley Falkow (Stanford Univ.), Emilio Garcia and Valdimir Motin (Lawrence Livermore Natl. Lab.), Jon Goguen (Univ. of

Massachusetts), B. Joseph Hinnebusch (NIH Rocky Mountain labs), Luther Lindler (Walter Reed Army Institute of Research), Hank Lockman (Battelle Memorial Institute), Kathleen McDonough (New York State Dept. of Health, David Axelrod Institute), Scott Minich (Univ. of Idaho), Mathew Nilles (Univ. of North Dakota), and Gregory Plano (Univ. of Miami, FL). These people do basic science studies that, in many cases where mutagenesis is used, generate literally thousands of still-more crippled strains, starting with the Pgm^+ Lcr^- or Pgm^- Lcr^+ parent *Y. pestis*. Such strains often exist only as colonies on plates or cultures in 96-well dishes while mass-screenings are done to sort them into categories and winnow down the pool into the few strains of interest. The number of strains in a lab using genetic methods to study plague pathogenesis is constantly changing – by large numbers -- with every transformation or mutagenesis that is done. This kind of work is the basis for discovery of new vaccine candidates and mechanisms of pathogenesis to target for novel antimicrobials. This work would be needlessly and seriously stifled by the inventory-controls over the numbers and fate of strains and other procedures stipulated in 42 CFR Part 1003 (Possession, Use and Transfer of Select Agents and Toxins; Interim Final Rule).

Finally, neither type of mutant is a likely source for use in bioterrorism/biowarfare. It would be much easier and more effective to obtain fully virulent strains from endemic foci in the U.S., South America, Russia, or states of the former Soviet Union than it would be to attempt to reconstruct a virulent strain from these mutants.

4. References

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